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β-catenin is a multifunctional protein that participates in cadherin-mediated cell-cell adhesion and in transduction of the Wnt growth factor signal that regulates development. At elevated cytoplasmic levels, β-catenin interacts with members of the LEF/TCF family of transcription factors and directly regulates gene expression. β-catenin also binds the adenomatous polyposis coli protein (APC). The tumor suppressor function of APC is suggested to depend in part on its ability to bind β-catenin and to facilitate β-catenin degradation by an unknown mechanism. The general purpose of this study is to better understand regulation of β-catenin, found aberrant in numerous cancers. We find that the cytoplasmic, signaling pool of β-catenin is regulated at the level of protein stability by the ubiquitin-proteasome degradation pathway. Extending the studies to include APC, we find that the ubiquitination and proteasomal degradation of β-catenin, and a serine kinase other than GSK-3β modulate the APC regulation of β-catenin.

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INTRODUCTION

Relevance and Nature of Problem

One in 8 American women will develop breast cancer in her lifetime. Despite the advances in detection and treatment of breast cancer, the mortality from breast cancer has not changed significantly over the last forty years. Breast cancer treatments significantly include radiation and chemotherapy. These regimens are acutely limited by the lack of ability to specifically target tumor cells. The emotional state of the patient which could be a critical factor in combating the disease is gravely debilitated by the psychosomatic trauma of these severe treatment procedures. Even those patients that survive, face the possibility of remission and an uncertain future. Unlike the survival after many other cancers, which tend to level off after 5 years, survival after diagnosis of breast cancer continues to decline. Even women who try to make preventive life-style changes cannot alter the most significant risk factors like age or family history. In this grim scenario, a basic understanding of the cellular processes underlying breast cancer is mandated before effective therapies can be developed or even attempted.

β-catenin is a multifunctional protein that primarily helps link the cadherins (at the adherens junctions) to the cytoskeleton. However, β-catenin is also a crucial signaling molecule that participates in differentiation and proliferation pathways. The *wnt* signaling pathway, known to reverse contact inhibition in mouse mammary cells *in vitro* and to cause mammary cancer in mice (7), results in increased levels of cytoplasmic β-catenin (8). *Wnt*-1 stimulation results in decreased activity of glycogen synthase kinase (GSK)-3β, that normally phosphorylates the tumor suppressor adenomatous polyposis coli (APC) gene product (8,9). When APC is not phosphorylated, it leads to the stabilization of β-catenin. The stable β-catenin interacts with the transcriptional activators LEF/TCF (10). The β-catenin-TCF/LEF complex translocates to the nucleus and effects gene expression (1,2) . The genes activated may include those that stimulate proliferation or antagonize apoptosis (11,12). And finally, stable forms of β-catenin by themselves are oncogenic (3,12,13). These observations strongly point towards the stability of cytoplasmic β-catenin as a "smoking gun" (12) linking cell adhesion and tumorigenesis. Thus, a strategy of down-regulating β-catenin could constitute a potential way of treating breast cancer. In this study, we investigate the regulation of cytoplasmic β-catenin.

Background

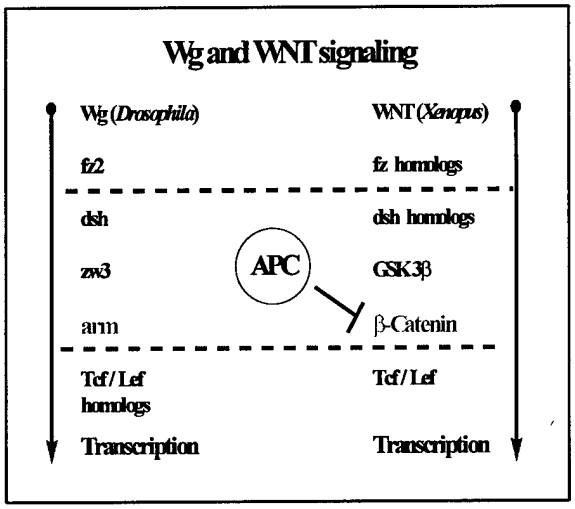
β- Catenin and breast cancer

Cells touch one another through a number of different surface molecules; among the most intriguing are the cadherins and their associated proteins (14). These proteins, in addition to maintaining adhesion of adult tissues, via the adherens junctions, are critical during development and tumorigenesis (15). Cadherin function has been shown to depend on several associated proteins, namely; α, β, and (plakoglobin) γ catenin (16). These molecules, link cadherins to the actin cytoskeleton and are probably involved in relaying cadherin-mediated-contact signals (17). The β-catenin/cadherin association requires serine phosphorylation of the cadherin molecule

(17). β–catenin is itself a substrate for tyrosine phosphorylation and can also act as a link between Growth factor receptors (such as the EGFR) and the adherens junction complex (18,19). Mutation of the β-catenin gene in mice, by homologous recombination, results in embryonal lethality. When the expression of E-cadherin and the catenins was analyzed in human breast carcinomas, lobular breast carcinomas showed disturbances of E-cadherin and catenins in a high frequency of cases (20). In ductal breast carcinomas (where E-cadherin is often unchanged), a high frequency of cases showed disturbance of alpha- and/or gamma-catenin expression. 50 % of cases with defects in E-cadherin and catenins had lymph node metastasis, whereas this number was low in cases with undisturbed cadherin/catenin expression (20).

A truncated stable form of β-catenin itself acts as an oncogene (9). The phosphorylation state of β-catenin can also influence the transformed phenotype (19,21). Further, cytoplasmic β-catenin associates with the tumor suppressor adenomatous polyposis coli (APC) gene product (19). Over-expression of APC results in the cell cycle being blocked at the G1/S boundary (19). Recent evidence indicating that the tumor suppressor effects of APC are dependent upon its ability to destabilize β-catenin, strongly argue the significance of β-catenin in the control of cell proliferation (5,22).

β-catenin is a signaling molecule



β-catenin participates in developmental patterning in *Xenopus* (23). Ectopic expression of β-catenin by mRNA injection into the ventral region of *Xenopus* embryos induces a secondary dorso-anterior body axis, giving rise to two heads, notochords, and neural tubes (24). Wnt-1, the vertebrate homologue of wingless is known to reverse contact inhibition in mouse mammary cells *in vitro* and to cause breast cancer in mice (24). Wnt-1 stimulation results in decreased activity of glycogen synthase kinase (GSK)-3β, that normally phosphorylates the tumor suppressor adenomatous polyposis coli (APC) gene product (5,8). When APC is not phosphorylated, it leads to the stabilization of β-catenin through an unknown mechanism. Now, β-catenin interacts with transcriptional activators LEF/TCF, translocates to the nucleus, and effects gene expression (10,25). The genes activated may include those that stimulate proliferation or antagonize apoptosis.

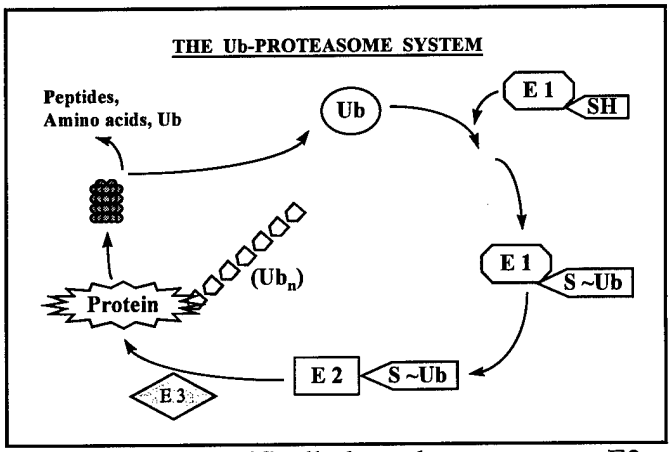
Taken together, these finding strongly argue the significance of β-catenin and its cytoplasmic levels in the integration of adhesion, differentiation and proliferation pathways. A clearer understanding of this crucial signaling pathway holds tremendous potential to offer realistic strategies to combat breast cancer.

β-catenin stability and APC

The Adenomatous polyposis Coli (APC) gene is a tumor suppressor, found mutated in most human colon cancers. APC directly binds β-catenin (26). APC is a part of the *wnt* signaling pathway, and when phosphorylated by GSK3β, down-regulates β-catenin levels. Cancer cells with mutant APC contain abnormally high levels of cytoplasmic β-catenin (4,5). Over-expression of APC blocks progression of the cell cycle from G_0 to the S phase (27). This observation suggests that loss APC activity (resulting in β-catenin stabilization) could lead to uncontrolled cellular proliferation. Indeed, the *wnt* signal, thought to inactivate APC, can cause cell proliferation in certain tissues. Although APC has been primarily studied in colon cancer, there is strong evidence that loss of heterozygosity at the APC locus may be involved in mammary tumors in humans. The Multiple intestinal neoplasia (Min) allele is a mutant allele of the murine APC locus. Min-/+ mice are predisposed not only to intestinal but mammary carcinoma as well (7). Among Min-/+ mice exposed to carcinogenic material, over 75% developed mammary tumors, while Min +/+ mice displayed no evidence of mammary tumors (7). These observations suggest that APC, by virtue of its ability to regulate β-catenin, can play an important role in predisposing breast tissue for further hyperplastic events (11).

The ubiquitin-proteasome pathway

The ubiquitin-proteasome pathway is involved in the processing and rapid degradation of many short-lived regulatory proteins. Mitotic cyclins, cyclin-dependent kinase inhibitors, the tumor suppressor p53, transcriptional activators NF-κB, v-jun, and v-fos are examples of proteins that are degraded by this highly specific pathway (28-31).



The ubiquitin pathway effects the degradation of proteins in two steps (28). First, multiple ubiquitin moieties are covalently attached to a target protein. Second, the multi-ubiquitinated protein is degraded by the 26S proteasome complex. Conjugation of ubiquitin moieties, to a substrate, is performed in a three-step process. Following activation of the C-terminal glycine of ubiquitin by enzyme E1, one of several E2 enzymes transfers the activated ubiquitin to the substrate that is specifically bound to an enzyme E3. E3 catalyzes the formation of an isopeptide bond between the activated glycine on the ubiquitin and ε-NH_2 group of a lysine residue in the substrate (or in the previously conjugated ubiquitin moiety). The E2 and E3 enzymes bind the substrate, and help transfer the ubiquitin moieties. There are dozens of genes, unrelated to each other, that encode E2 and E3 enzymes. The specificity of the ubiquitin pathway is thought to reside in the E3 enzymes (28). Following targeting (e.g. phosphorylation, as in the case of NF-κB) and multi-ubiquitination, the substrate protein is rapidly degraded by a large multi-subunit structure called the proteasome.

PURPOSE

The general aim of this investigation is to study the regulation of cytoplasmic β-catenin stability, and the involvement of the tumor suppressor APC in this process.

SPECIFIC AIMS

Aim 1. To test the hypothesis that cytoplasmic β-catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

Aim 2. To establish an *in vitro* cell-free model to study β-catenin ubiquitination and degradation.

Aim 3. To test the hypothesis that the ubiquitin/proteasome pathway modulates APC regulation of β-catenin and β-catenin-LEF signaling.

Aim 4. To test the hypothesis that lithium, an inhibitor of GSK3β, represses the ability of APC to regulate β-catenin and β-catenin-LEF signaling.

METHODS

Reagents, Antibodies, and Cells:

ALLN (*N*-acetyl-Leu-Leu-norleucinal), ALLM (*N*-acetyl-Leu-Leu-methional), Lactacystin, and MG-132 were purchased from Calbiochem. GF-109203X was purchased from Boehringer Mannheim. Ro31-8220 was a gift from Dr. Robert Glazer. The monoclonal anti-β-catenin antibody (Clone#14) and the anti-FLAG™ antibody were purchased from Transduction Labs and Kodak, respectively. Affinity purified rabbit polyclonal anti-APC2 and anti-APC3 antibodies (125) were generously provided by Dr. Paul Polakis (Onyx Pharmaceuticals, CA). The LEF and AP-1 reporters were gifts from Dr. Hans Clevers and Dr. Powell Brown, respectively. Affinity purified FITC- conjugated goat anti-rabbit and Texas Red- conjugated goat anti-mouse antibodies were purchased from Kirkegaard and Perry Laboratories. The cancer cell lines were acquired from the ATCC and maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 1% penicillin/streptomycin.

To test the hypothesis that cytoplasmic β-catenin is regulated at the level of stability by the ubiquitin-proteasome pathway:

- The effect of proteasomal inhibitors on cytoplasmic β-catenin stability was tested. The breast cancer cell line SKBR3 (APC+/+) and the colon cancer cell line SW480 (APC-/-) were used in this experiment. Cells were treated with the proteasomal inhibitors (peptidyl aldehydes) ALLN and Lactacystin (32) for 12 hr. Cells were lysed in a hypotonic lysis buffer and dounce homogenized, clarified in a ultracentrifuge (100,000g for 1 hr) to yield the S100 cytoplasmic fraction (free of membranous components). To obtain cytoplasmic fractions including membrane vesicles, the dounced lysate were clarified in a table-top microfuge (10,000g for 10 min).

- The half-life of β-catenin in E36ts20 cells (33), that harbor a temperature sensitive E1 enzyme, was monitored at permissive and non-permissive temperatures
- SKBR3 cells were transient transfected with His_6-tagged ubiquitin (34) and HA-tagged β-catenin. Cells were treated with/without proteasomal inhibitors, ubiquitinated proteins were purified with Ni-NTA columns (34), and Western blotted with anti-HA antibody (helps distinguish from native β-catenin).

To establish an *in vitro* cell-free model to study β-catenin ubiquitination and degradation.
In vitro ubiquitination and degradation assays will be established according to published protocols (29,35,36):

- Recombinant β-catenin was generated in a combined *in vitro* transcription-translation system (Promega). mRNA synthesized from 2 μg of template DNA was used in a 100 μl translation reaction mixture containing 50 μl of rabbit reticulocyte lysate (RRL).
- Conjugation assays (29,35) are performed essentially as described by Dr. Ciechanover (29,30,32). Briefly, the reaction mixture (30 μl) consisted of 1 μl of either programmed RRL containing the HA-tagged β catenin or unprogrammed RRL, 10 μl SKBR3 hypotonic lysate (5-6 mg/ml) or, 20 ng E1, 20 ng E2 (UbcH5b) and, either 40 ng E3 (E6-AP)(recombinant enzymes were kindly provided by Dr. Allan Weissman) or 40 ng of various APC deletion constructs. 5 μg ubiquitin, 40 mM Tris HCl (pH 7.6), 5 mM $MgCl_2$, 2mM DTT, 0.5 μg of ubiquitin aldehyde (kindly provided by Dr. Keith Wilkinson), 5mM ATPγS, 10mM Phosphocreatine, and 5 units Phosphocreatine Kinase, were also included in the reaction mixture. In Assays without ATPγS, 20 mM EDTA, 0.5 μg of hexokinase and 10 mM 2-deoxyglucose substituted for ATPγS. Ubiquitination assays were performed for a 1 hr period at 25°C. Degradation assays included ATP (instead of ATPγS, which cannot be used by the proteasome but can be utilized by the ubiquitination enzymes), and were performed at 37°C for 2 hr. Following incubation, reaction mixtures are resolved by Tris-glycine SDS-PAGE (4-12%).
- Western blotting was performed using anti-HA (BabCo) and anti-β-catenin antibodies (Transduction labs).

To test the hypothesis that the tumor suppressor adenomatous polyposis coli (APC) gene product is involved in the ubiquitin-proteasome pathway that regulates β-catenin:

- *In vitro* ubiquitination assay with SW480 (APC-/-) cytoplasmic fraction and recombinant APC constructs with/without the HECT consensus.
- Test the effects of alkylating agents (alkylating agents like N-ethyl maleimide (NEM) block the free cysteine residues necessary for the thiol-ester transfer of ubiquitin) (36) on the ligase activity of recombinant APC
- Transient transfection of SW480 (APC-/-) cells with APC (4), monitor cytoplasmic β-catenin levels in response to proteasomal inhibitors.

SW480 cells (APC-/-) were co-transfected with a vector encoding His_6-tagged ubiquitin (34) and vector encoding APC 25 (4) (the smallest construct including the free cysteine consensus) or vector alone, using Lipofectamine™ (GIBCO) according to manufacturer's instructions. 24 hr

after transfection, the cells were treated with the proteasome-specific inhibitor, Lactacystin for 6hr. Ubiquitinated proteins were purified by Ni-NTA chromatography, and Western blotted with anti-β-catenin antibody

Liposome-mediated transfections and LEF-Luciferase reporter assays:

The SW480 and CACO-2 colon cancer cell lines used in these studies have been traditionally transfected using lipid-mediated protocols. Following established protocols, cells were seeded in 12 well-plates at 1 x 10^5 cells per well. 24-36 h later, cells were transiently transfected with 1 μg of empty vector or APC constructs, 0.5 μg of the LEF-reporters pTOPFLASH (optimal motif) or pFOPFLASH (mutated motif) (148), and 0.008 μg pCMV-Renilla Luciferase (Promega), per well, using Lipofectamine-Plus reagent according to manufacturers instructions (GIBCO-BRL) for 5 h. In experiments using increasing concentrations of APC, and in other experiments involving different transfection-dosages, the total amount of transfected DNA was maintained constant. In studies designed to monitor the effect of APC on β-catenin protein, 0.3μg FLAG-tagged wt β-catenin was co-transfected with 0.6μg empty vector or APC constructs. This approach facilitated analysis of only the transfected cells, using anti-FLAG antibodies.
Cells were treated with indicated levels of the inhibitors for 12-24 h. Luciferase activity was monitored using the Dual-Luciferase Assay System (Promega). The experimental LEF-Luciferase reporter activity was controlled for transfection efficiency, and potential toxicity of treatments using the constitutively expressed Renilla Luciferase. The experiments were repeated at least three times, with each treatment repeated in triplicate. Error bars represent standard deviation. The question of promoter specificity was addressed using an unrelated AP-1 reporter. The AP-1 reporter assays were performed as described above for the LEF-reporters.

Immunofluorescent staining:

Cells were grown on cover-slips, washed with PBS, and fixed in ice-cold methanol for 5 min at -20°C. In co-staining experiments, β-catenin was detected first using 1:50 dilution of a mouse monoclonal antibody (Transduction Labs) followed by Texas Red-conjugated goat anti-mouse antibodies (Kirkegaard and Perry Labs) at 1:75 dilution. Next, APC was detected using a 1:1 mixture of affinity-purified rabbit polyclonal antibodies APC2 and APC3 (at 1:100 dilution) (125) followed by fluorescein-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Labs) at 1:50 dilution. In experiments where FLAG-tagged β-catenin was co-transfected with APC, anti-FLAG™ antibodies (Kodak) were used to detect the β-catenin.
Following immunostaining, transfected cells in 30 fields were counted and scored for APC staining versus presence or absence of β-catenin, with or without various inhibitors.

RESULTS AND DISCUSSION

Cytoplasmic β-catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

1. Results indicate that treatment of SKBR3 cells with proteasomal inhibitors ALLN and Lactacystin (32) result in the accumulation of high-molecular weight, β-catenin-ubiquitin conjugates in the cytoplasm (Addenda; Fig. 1).
2. E36ts20 cells harboring a thermolabile Ubiquitin activating (E1) enzyme (33), when grown at the non-permissive temperature (39.5^{0}C) accumulate β-catenin (half-life is extended, compared to cells grown at the permissive temperature; 30^{0}C) (Fig. 2).
3. In a more direct approach, SKBR3 cells were co-transfected with a vector encoding His_6-tagged ubiquitin (34) and a vector encoding HA-tagged β-catenin (Fig. 3). 48 hr after transfection, the cells were treated with the proteasome specific inhibitor, Lactacystin, for 6 hr. Ubiquitinated proteins were purified by Ni-NTA chromatography (34) and Western blotted with anti-HA antibody (Fig. 4). β-catenin was found to accumulate as high- molecular weight ubiquitinated conjugates, in response to the proteasome-specific inhibitor Lactacystin.
These observations demonstrate that cytoplasmic β-catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

β-catenin ubiquitination and degradation *in vitro*.

In vitro ubiquitination and degradation assays were established according to published protocols (29,35,36) and with the help of our collaborators Drs. Aaron Ciechanover and Allan Weissman.
1. Cytosol that included membrane and/or particulate material was able to ubiquitinate β-catenin more efficiently than a S100 preparation that lacked them (Fig. 5).
2. Cytosol extracted from cells in different phases of the cell cycle, strikingly varied in their ability to ubiquitinate β-catenin (Fig. 6).
3. The most efficient ubiquitination activity was observed in the extracts from cells in M-phase (Fig. 6).

APC is involved in the ubiquitin-proteasome pathway that regulates β-catenin.

1. Recombinant APC constructs help degrade β-catenin in SW480 (APC-/-) cell lysate, *in vitro*. APC constructs 4 and 25 (Fig.8) include a free-cysteine-HECT consensus of ubiquitin ligases, whereas APC 3 does not. Lane 6; In the presence of a proteasomal inhibitor, a high molecular weight band is observed, which probably is a mono-ubiquitinated β-catenin (Fig.10).
2.Anti-APC antibodies and N-ethyl Maleimide (NEM) (4) inhibit the ubiquitination of β-catenin, *in vitro* (Fig. 9).
3. In SW480 cells transfected with APC (Fig. 11), β-catenin was found to be stabilized as ubiquitinated conjugates, in response to lactacystin, a specific proteasomal inhibitor. In cells transfected with vector alone, the proteasomal inhibitor had no effect. However, in Lane 6, the APC 3 transfection did not seem to have worked well in this particular experiment.

In vitro reconstitution experiments with recombinant β-catenin, E1, E2, and various recombinant APC fragments (with/without the HECT consensus) have proved to be elusive. Considering the increasing number of similarities between the regulation of IκB and β-catenin, it is tempting to speculate that β-catenin ubiquitination occurs in a multi-protein complex that includes kinases, ubiquitin conjugating enzymes, and co-factors. In fact, during our efforts to study β-catenin ubiquitination *in vitro* using cell-free systems, we observed that cell extracts which included membranous and particulate material sustained the most robust ubiquitination reactions (data not shown). Further i*n vitro* reconstitution experiments designed to explore a more direct role in β-catenin ubiquitination suggested the requirement of key components other than GSK3β, and APC (Easwaran, unpublished observations). During the course of this study, there has been an explosion of data describing increasing numbers of novel proteins including; axin, conductin, and potentially cullin, as regulators of β-catenin signaling. One model describes axin and/or conductin to function downstream of APC because overexpression in cells with mutant, non-functional APC reduces LEF-reporter activity and β-catenin levels. Another study describes a direct interaction between conductin and GSK3β, APC, and β-catenin. However, a form of conductin that is unable to bind APC can still induce β-catenin degradation in cells expressing non-functional APC. In contrast, a form of axin missing the APC binding region acts as a dominant negative and results in axis duplication in Xenopus, an effect attributed to increased β-catenin. The data reviewed above indicate that the APC- dependent or independent regulation of β-catenin signaling is an extremely complicated process that requires further study.

Proteasomal degradation modulates APC regulation of β-catenin - LEF signaling

Fig. 12 shows that the APC mediated down-regulation of β-catenin-LEF signaling is reversed by a panel of proteasomal inhibitors including ALLN, Lactacystin, and MG-132, but not DMSO (vehicle) alone or ALLM. In the same experiment β-catenin protein levels were monitored by immunoblot. Changes in LEF-reporter activity were paralleled by changes in β-catenin protein. (Fig. 13). These observations demonstrate that APC mediated down-regulation of β-catenin-LEF signaling requires proteasomal degradation.

APC can down-regulate signaling induced by Wt β-catenin but not by a non-ubiquitinatable, S37A mutant β-catenin.

Mutation of a single serine residue (S37A) within the ubiquitination targeting sequence (UTS) prevents β-catenin ubiquitination (Orford et al., 1997). Non-ubiquitinated β-catenin is not a substrate for the proteasome and thus accumulates in the cytoplasm. If APC regulates β-catenin-LEF signaling by targeting β-catenin for proteasomal degradation, then it should not be able to regulate the non-ubiquitinatable S37A mutant β-catenin induced LEF signaling.
Wt or S37A mutant β-catenin constructs were co-transfected with APC 25 or 3 and the LEF-reporters, into SW480 cells. The more stable S37A mutant stimulated β-catenin-LEF signaling to greater levels than wt β-catenin. APC down-regulated wt β-catenin but not the S37A mutant β-catenin induced LEF signaling (Fig. 14). Changes in LEF-reporter activity were paralleled by changes in β-catenin protein. (Fig. 15).

Lithium, an inhibitor of GSK3β, does not significantly alter the ability of exogenous APC down-regulate β-catenin.

Physiologically effective concentrations of Li^+ specifically and reversibly inhibit GSK-3β activity *in vitro* and *in vivo*, and mimic the effects of Wnt signaling on β-catenin in mammalian cells. Treatment of the breast cancer cell lines SKBR3 and HBL100 with lithium results in the accumulation of the cytoplasmic, signaling pool of β-catenin. The exact mechanism by which inhibition of GSK-3β leads to stabilization of β-catenin is unknown. However, Rubinfeld et al., (1996) have shown that the APC protein is phosporylated by GSK-3β, and that this phosphorylation event is linked to β-catenin stabilization.

We tested the hypothesis that Li^+ can inhibit the ability of APC to down-regulate β-catenin - LEF signaling. The colon cancer cell lines SW480 and CAC02 were transfected with empty vector or wtAPC, and treated with NaCl or LiCl. Lithium causes a four-fold increase in LEF-reporter activity in cells transfected with vector alone, most probably by a mechanism independent of APC since the cell lines used do not contain functional APC (Fig. 16). Changes in LEF-reporter activity were paralleled by changes in β-catenin protein. (Fig. 17). Interestingly, lithium does not significantly alter the ability of wt APC to down-regulate LEF-reporter activity. In light of recent additions to the Wnt signaling pathway, the effect of lithium and inhibition of GSK-3β on β-catenin-LEF signaling and the precise role of APC in this process need to be evaluated in further detail.

CONCLUSIONS

1. Cytoplasmic β-catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.
2. The ubiquitin/proteasome pathway modulates APC regulation of β-catenin protein and β-catenin-LEF signaling.

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ADDENDA

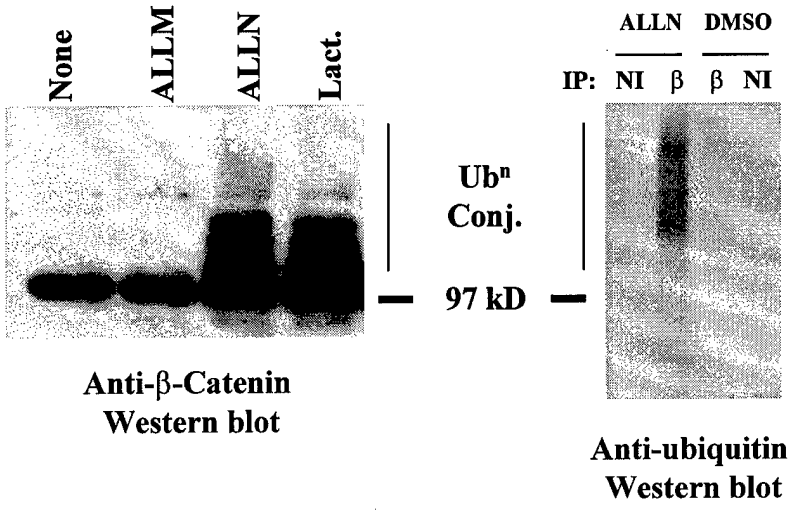
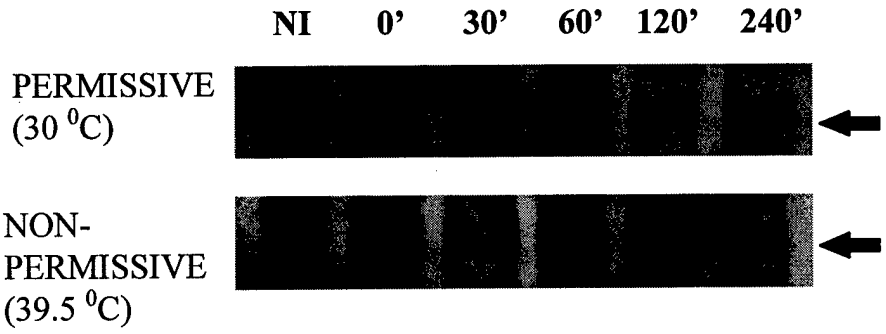


Figure 1. β-catenin accumulates as high- molecular weight ubiquitinated conjugates, in response to the proteasomal inhibitor ALLN and Lactacystin. ALLM is the negative control.
In the right panel, β-catenin was immunoprecipitated with a C-terminal monoclonal antibody from a NP-40 lysate, and Western blotted with anti-ubiquitin antibody. NI: Non-Immune, β: β-catenin.



AUTORADIOGRAPH OF IMMUNOPRECIPITATED [^{35}S] β CATENIN

Figure 2. Pulse chase in E36ts20 cells harboring a thermolabile Ubiquitin activating (E1) enzyme. When grown at the non-permissive temperature, the half-life of β-catenin is extended.

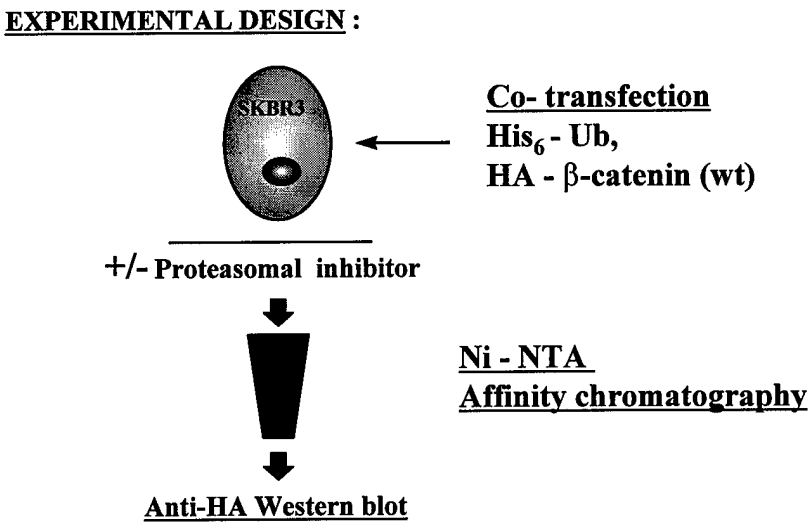


Figure 3. Experimental design to test the hypothesis that cytoplasmic β-catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

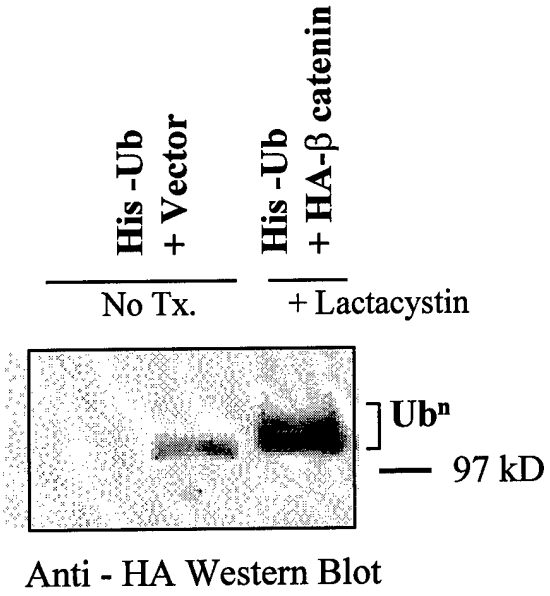


Figure 4. β-catenin accumulates as high- molecular weight ubiquitinated conjugates, in response to the proteasomal inhibitor Lactacystin.

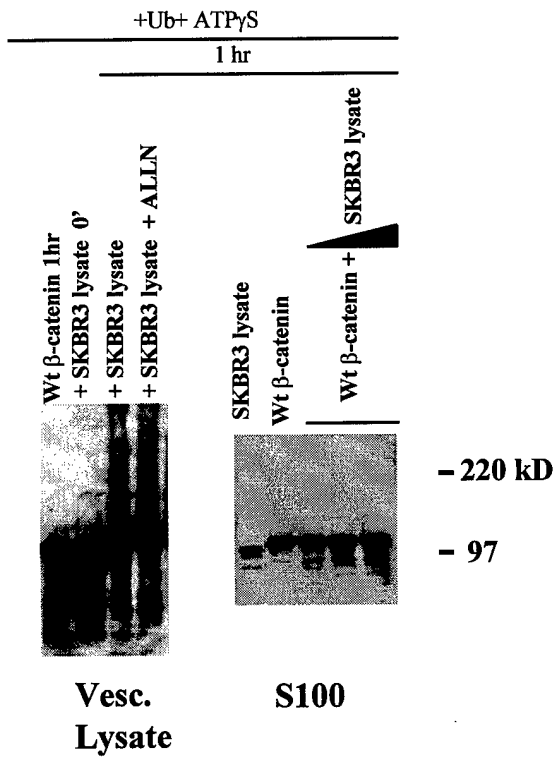


Figure 5. I*n vitro* β-catenin ubiquitination assay. Cytosol that included membrane material (vesicular lysate) ubiquitinated β-catenin more efficiently than a S100 preparation that lacked them.

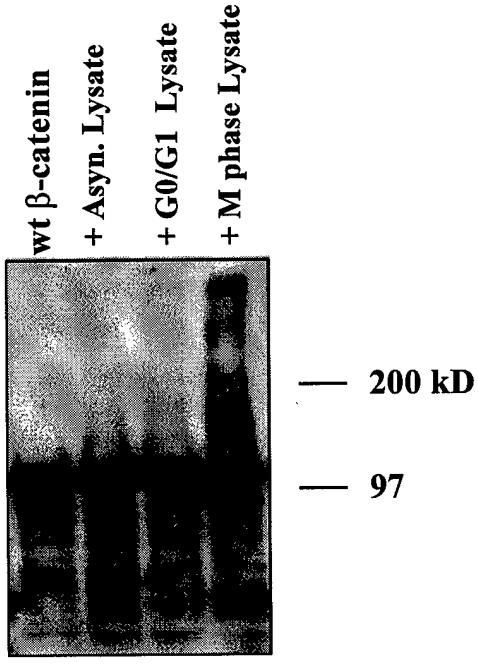


Figure 6. I*n vitro* β-catenin ubiquitination assay. Cytosol extracted from cells in different phases of the cell cycle, strikingly varied in their ability to ubiquitinate β-catenin. The most efficient ubiquitination activity was observed in the extracts from cells in M-phase.

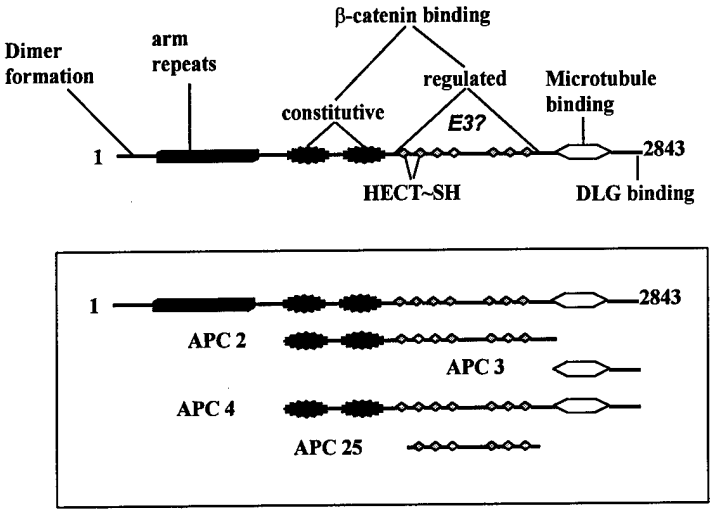
Figure 7.

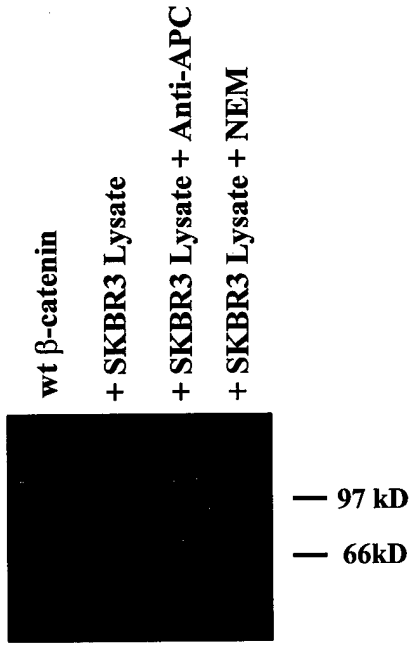
The second β-catenin binding repeat of APC contains a free cysteine consensus region present in the HECT family ubiquitin ligases

	~ SH
	L P L ▼ V V Y
HECT domain	P L X X X X C X X X S X L X X F
Human APC	P L X X X X C X X X S X L X X F
	1387
Mouse APC	P L X X X X C X X X S X L X X F
Xenopus APC	P L X X X X C X X X S X L X X F

Figure 8.

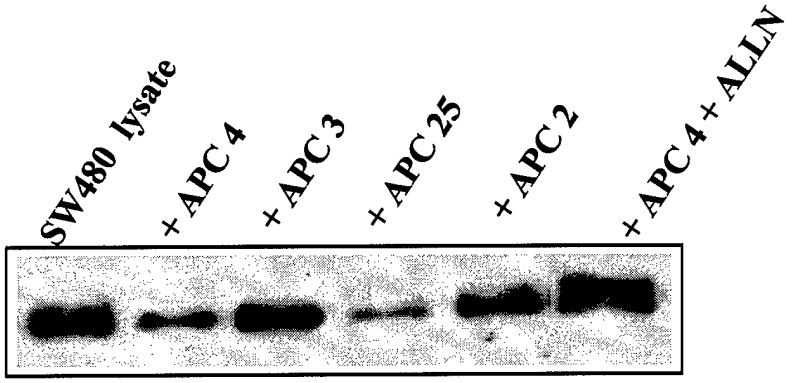
APC Functional Domains and Constructs





Anti- β-Catenin Western blot

Figure 9. *In vitro* degradation assay. Anti-APC antibodies and N-ethyl maleimide (NEM) inhibit the *in vitro* degradation β-catenin



Anti β-catenin Western blot

Figure 10. I*n vitro* β-catenin ubiquitination assay. Recombinant APC constructs help degrade β-catenin in SW480 (APC-/-) cell lysate, *in vitro*. Constructs 4, 25, and 2 include a free-cysteine-HECT consensus of ubiquitin ligases. APC 3 does not. Lane 6; In the presence of a proteasomal inhibitor, a high molecular weight band is observed, which probably is a mono-ubiquitinated β-catenin

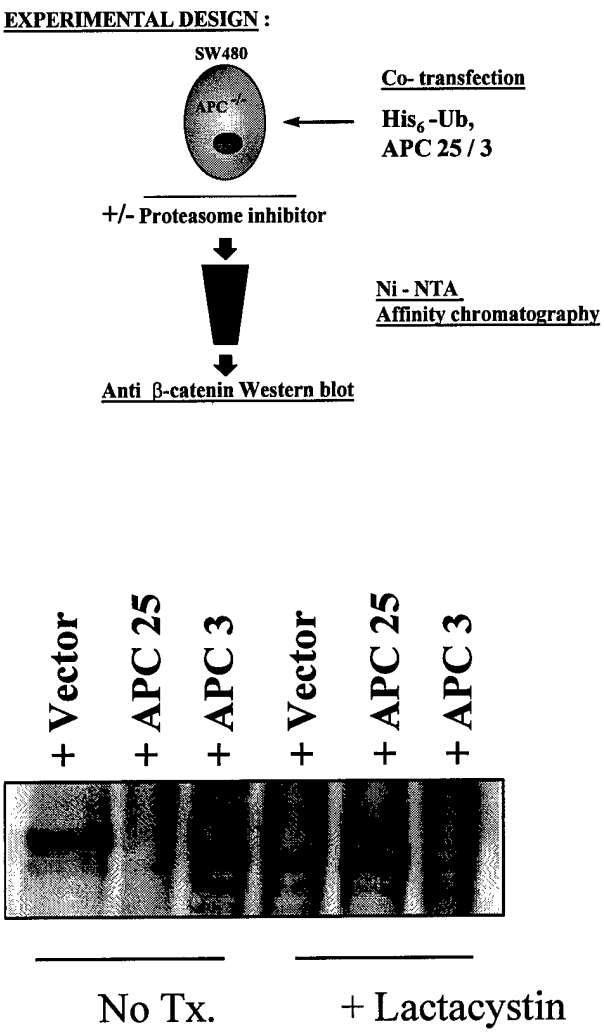


Figure 11. SW480 cells (APC-/-) were co-transfected with a vector encoding His_6-tagged ubiquitin and a vector encoding APC 25 (the smallest construct including the free cysteine consensus) , APC 3, or vector alone. 24 hr after transfection, cells were treated with the proteasome-specific inhibitor, Lactacystin for 6 hr. Ubiquitinated proteins were purified by Ni-NTA chromatography, and Western blotted with anti-β-catenin antibody. In cells transfected with APC 25, β-catenin was found to be stabilized as ubiquitinated conjugates, in response to a specific proteasomal inhibitor. In cells transfected with vector alone, the proteasomal inhibitor had no effect probably because the native ubiquitination machinery is not functional. Lane 6; the APC 3 transfection did not seem to have worked well in this particular experiment.

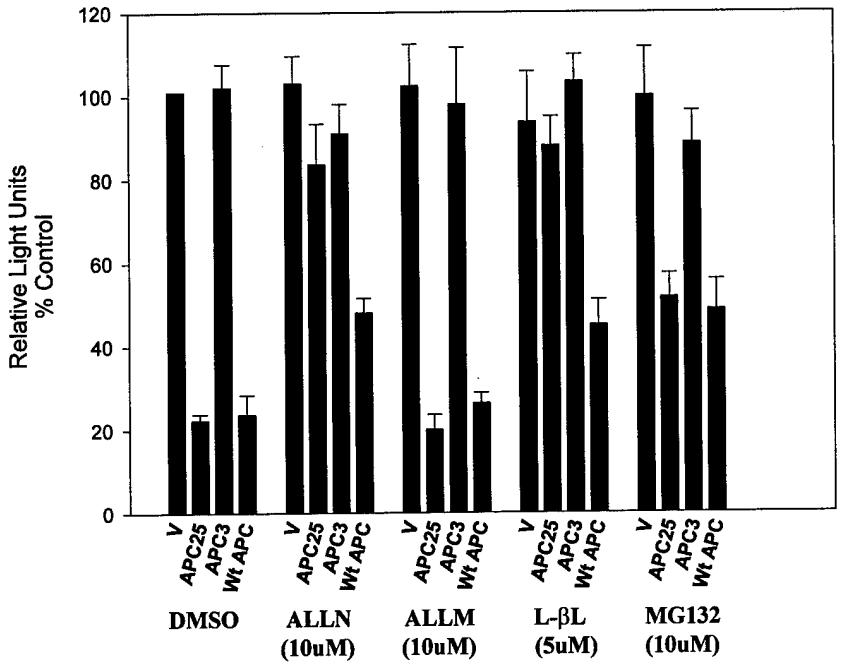


Figure 12. APC mediated down-regulation of β-catenin-LEF signaling is reversed by proteasomal inhibitors. SW480 cells were transiently transfected with various APC constructs, using Lipofectamine-Plus reagent (GIBCO BRL). 12 hr post-transfection, the cells were treated with proteasomal inhibitors ALLN, Lactacystin-β lactone, and MG-132, or with DMSO (vehicle) and ALLM (Calpain inhibitor II) for 12 hr. β-catenin-LEF signaling was assayed using the LEF-reporters pTOPFLASH (and pFOPFLASH; data not shown). Raw data was normalized for transfection efficiency and potential toxicity of treatments, using pCMV-Renilla luciferase and the Dual Luciferase Assay System (Promega). The experiment was repeated at least three times, with each treatment repeated in triplicate. Error bars represent Standard Deviation.

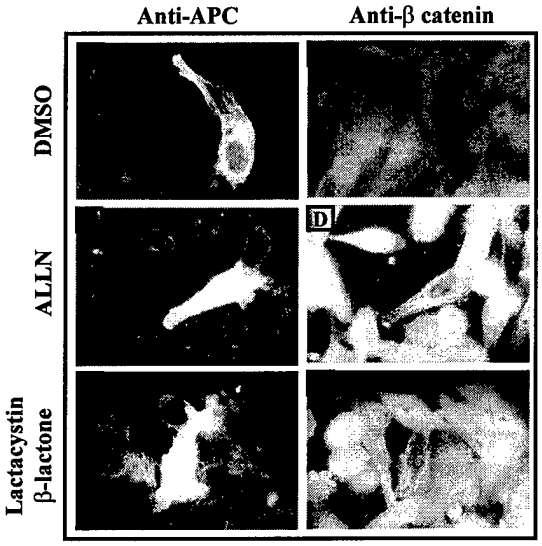


Figure 13. APC mediated down-regulation of β-catenin protein is reversed by proteasomal inhibitors. SW480 cells were transfected with wt APC and treated with DMSO (A and B), or 10 μM ALLN (C and D) or 5 μM Lactacystin *β*– lactone (E and F). Double-immunofluorescent staining for APC (A, C, and E) and β-catenin (B, D, and F).

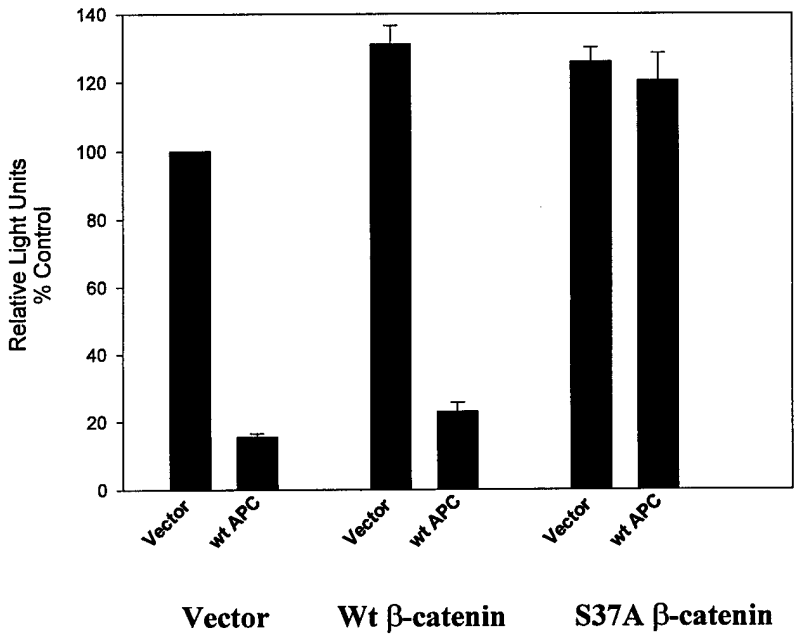


Figure 14. APC down-regulates LEF signaling induced by wt β-catenin but not by the non-ubiquitinatable S37A mutant β-catenin. SW480 cells were transfected with empty vector or FLAG tagged -wt β-catenin or -S37A β-catenin, and empty vector or wt APC constructs, LEF-reporters and pCMV-Renilla luciferase. 24 hr post-transfection LEF-reporter activity was monitored using the Dual-Luciferase Assay System (Promega).

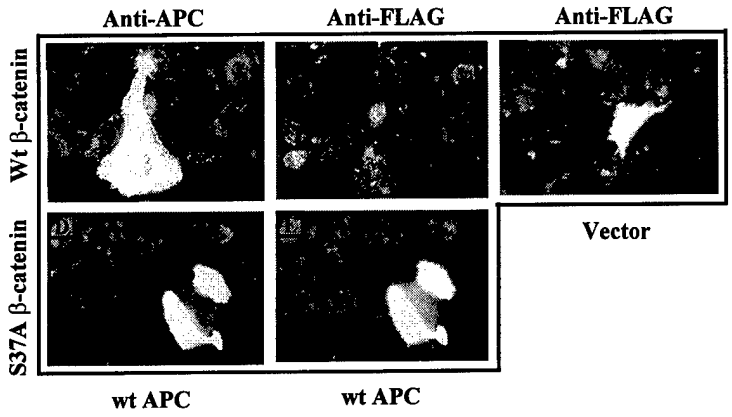


Figure 15. APC down-regulates wt β-catenin but not the non-ubiquitinatable S37A mutant β-catenin protein. SW480 cells were transfected with FLAG tagged -wt β-catenin (A, B and C) or -S37A β-catenin (D and E), and wt APC constructs (A, B, D, and E) or empty vector (C). Double-immunofluorescent staining for APC (A and D) and β-catenin (B, C, and E). The tranfected FLAG-tagged β-catenin was detected using anti-FLAG antibodies (Kodak).

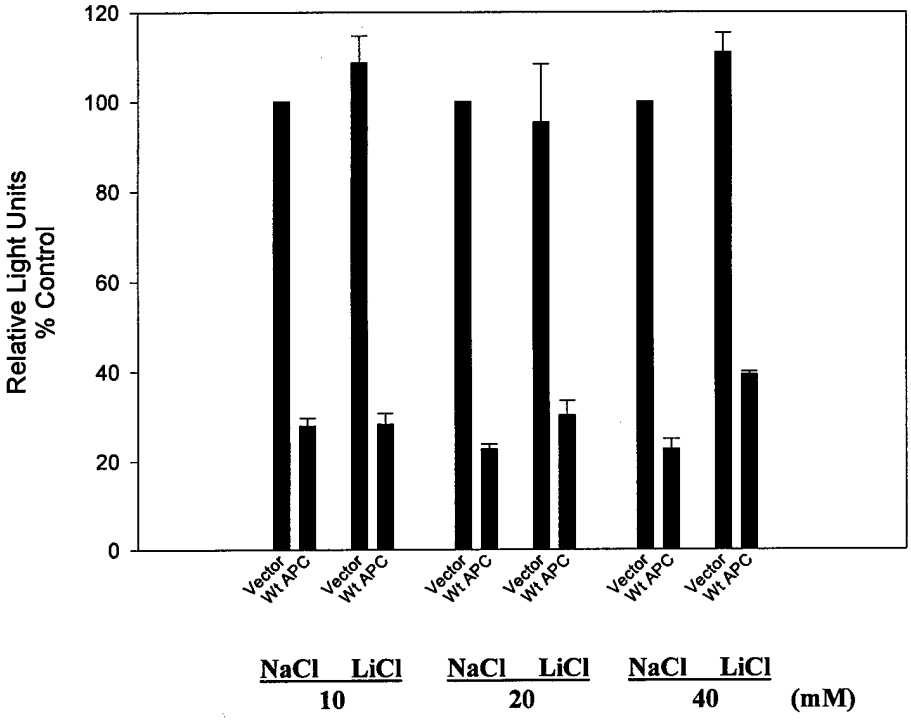


Figure 16. Lithium, an inhibitor of GSK-3β, does not significantly alter the ability of exogenous wt APC to down-regulate LEF-reporter activity. SW480 cells were transfected with empty vector or wt APC, LEF-reporters and pCMV-Renilla luciferase. Various concentrations of NaCl or LiCl were added immediately after transfection, to assure GSK-3β repression. 24 hr later LEF-reporter activity was monitored using the Dual Luciferase Assay system (Promega).

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The Ubiquitin-Proteasome Pathway and Serine Kinase Activity Modulate Adenomatous Polyposis Coli Protein-mediated Regulation of β-Catenin-Lymphocyte Enhancer-binding Factor Signaling*

(Received for publication, January 21, 1999)

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The tumor suppressor function of the adenomatous polyposis coli protein (APC) depends, in part, on its ability to bind and regulate the multifunctional protein, β-catenin. β-Catenin binds the high mobility group box transcription factors, lymphocyte enhancer-binding factor (LEF) and T-cell factor, to directly regulate gene transcription. Using LEF reporter assays we find that APC-mediated down-regulation of β-catenin-LEF signaling is reversed by proteasomal inhibitors in a dose-dependent manner. APC down-regulates signaling induced by wild type β-catenin but not by the non-ubiquitinatable S37A mutant, β-catenin. Bisindoylmaleimide-type protein kinase C inhibitors, which prevent β-catenin ubiquitination, decrease the ability of APC to down-regulate β-catenin-LEF signaling. All these effects on LEF signaling are paralleled by changes in β-catenin protein levels. Lithium, an inhibitor of glycogen synthase kinase-3β, does not alter the ability of APC to down-regulate β-catenin protein and β-catenin-LEF signaling in the colon cancer cells that were tested. These results point to a role for β-catenin ubiquitination, proteasomal degradation, and potentially a serine kinase other than glycogen synthase kinase-3β in the tumor-suppressive actions of APC.

Mutations in the tumor suppressor adenomatous polyposis coli (APC)[1] gene are responsible for tumors that arise in both familial adenomatous polyposis and sporadic colon cancers (1–7). APC mutations are almost always truncating, giving rise to proteins lacking C termini (6, 8, 9). Efforts to understand how these mutations contribute to cancer have focused on the ability of APC to bind and subsequently down-regulate the cytoplasmic levels of β-catenin (10–13).

β-Catenin is a multifunctional protein that participates in cadherin-mediated cell-cell adhesion and in transduction of the Wnt growth factor signal that regulates development (14, 15). Activation of the Wnt growth factor signaling cascade results in the inhibition of the serine/threonine kinase, GSK-3β, and in response, β-catenin accumulates in the cytoplasm (16–18). At elevated cytoplasmic levels, β-catenin translocates to the nucleus, interacts with the high mobility group box transcriptional activator lymphocyte enhancer-binding factor (LEF)/T-cell factor, and directly regulates gene expression (19–22). Mutations that stabilize β-catenin protein are likely to be oncogenic, although this has not been proven directly (23).

The mechanism of APC-mediated β-catenin regulation is unknown. Recently, β-catenin was shown to be regulated at the level of protein stability via proteasomal degradation (24, 25). Proteins targeted for degradation by the ubiquitin-proteasome system are first tagged with multiple copies of the small protein ubiquitin by highly regulated ubiquitination machinery (27). Polyubiquitinated proteins are recognized and rapidly degraded by the proteasome, a large multisubunit proteolytic complex. Proteasomal degradation plays a critical role in the rapid elimination of many important regulatory proteins, *e.g.* cyclins and transcriptional activators like NFκB-IκB (28). Proteins regulated via proteasomal degradation can be specifically studied using the well characterized proteasome-specific peptidyl-aldehyde inhibitors (29, 30).

APC-mediated tumorigenesis might depend, in part, on its ability to regulate β-catenin signaling (26). In this report, we show that the ubiquitin-proteasome pathway and the activity of a serine kinase other than GSK-3β modulate APC-mediated regulation of β-catenin-LEF signaling.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Cells—ALLN, ALLM, lactacystin-β lactone, and MG-132 were purchased from Calbiochem. GF-109203X was purchased from Roche Molecular Biochemicals. Ro31-8220 was a gift from Dr. Robert Glazer. The monoclonal anti-β-catenin antibody (Clone 14) and the anti-FLAG™ antibody were purchased from Transduction Laboratories, Lexington, KY and Eastman Kodak Co., respectively. Affinity-purified rabbit polyclonal anti-APC2 and anti-APC3 antibodies (12) were generously provided by Dr. Paul Polakis (Onyx Pharmaceuticals). Affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse antibodies were purchased from Kirkegaard and Perry Laboratories. The SW480 and CACO-2 colon cancer cell lines were acquired from the ATCC and maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 1% penicillin/streptomycin.

Transfections and LEF-Luciferase Reporter Assays—Cells were seeded in 12-well plates at 1×10^5 cells/well. The following day cells were transiently transfected with 1 μg of APC constructs and 0.4 μg of the LEF reporter, pTOPFLASH (optimal motif), or pFOPFLASH (mutant motif) (31), and 0.008 μg of pCMV-*Renilla* luciferase (Promega) per well, using LipofectAMINE-Plus reagent according to the manufacturer's instructions (Life Technologies, Inc.) for 5 h. In experiments designed to monitor the effect of APC on β-catenin protein, 0.3 μg of FLAG-tagged WT or S37A β-catenin (25) was cotransfected with 0.6 μg of empty vector or APC constructs. This approach facilitated analysis of only the transfected cells, using anti-FLAG antibodies.

Cells were treated with indicated levels of the inhibitors for 12–24 h. Luciferase activity was monitored using the dual luciferase assay sys-

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[1] The abbreviations used are: APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase-3β; LEF, lymphocyte enhancer-binding factor; ALLN, *N*-acetyl-Leu-Leu-norleucinal; ALLM, *N*-acetyl-Leu-Leu-methional; WT, wild type; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DAG, diacylglycerol; NKκB, nuclear factor κB; IκB, inhibitor of NFκB.

tem (Promega). The experimental LEF-luciferase reporter activity was controlled for transfection efficiency and potential toxicity of treatments using the constitutively expressed pCMV-*Renilla* luciferase. The specificity of APC-mediated effects on LEF reporters was confirmed using pFOPFLASH, which harbors mutated LEF binding sites (31), and an unrelated AP-1 reporter (32).

Immunological Procedures—Double immunofluorescent staining for APC and β-catenin was performed according to Munemitsu *et al.* (11, 40). In experiments where FLAG-tagged β-catenin was cotransfected with APC, anti-FLAG™ antibodies (Kodak) were used to detect the exogenous β-catenin.

RESULTS AND DISCUSSION

APC-mediated Down-regulation of β-Catenin-LEF Signaling Is Reversed by Proteasomal Inhibitors—In the SW480 colon cancer cell line, which produces only a mutant APC protein containing amino acids 1–1337 of the complete 2843-amino acid sequence, overexpression of WT APC or deletion construct APC 25 (amino acids 1342–2075), but not APC 3 (amino acids 2130–2843) (Fig. 1*A*), can effect a posttranslational down-regulation of β-catenin (11, 26). We tested the hypothesis that APC effects the down-regulation of β-catenin-LEF signaling by targeting β-catenin for proteasomal degradation. SW480 cells were transiently transfected with various APC deletion constructs (Fig. 1*A*) and treated with proteasomal inhibitors, and β-catenin-LEF signaling was assayed using LEF reporters (31). Fig. 1*B* shows that the APC-mediated down-regulation of β-catenin-LEF signaling is reversed by a panel of proteasomal inhibitors including ALLN, lactacystin-β lactone, and MG-132, but not Me_2SO (vehicle) or ALLM (calpain inhibitor II), that effectively inhibits calpain proteases but has a 100-fold lower potency as a proteasomal inhibitor. The specificity of APC-mediated effects on LEF reporters was confirmed using pFOPFLASH, which harbors mutated LEF binding sites, and an unrelated AP-1 reporter, neither of which was influenced by APC (31, 32). The proteasomal inhibitor ALLN reverses the APC- mediated down-regulation of β-catenin-LEF signaling in a dose-dependent manner (Fig. 1*C*). The effects of APC 25 can be completely reversed by the proteasomal inhibitor ALLN, and the effects of WT APC can be restored to 50–60% of control values. However, the full-length WT APC construct, and not the APC 25 deletion construct, was used for all immunostaining experiments because it was more physiologically relevant (incorporating all the functional domains). SW480 cells were transfected with empty vector or WT APC and were treated with Me_2SO (vehicle) or the proteasomal inhibitors ALLN or lactacystin-β lactone. Double immunofluorescent staining for APC (Fig. 2, *A*, *C*, and *E*) and β-catenin (Fig. 2, *B*, *D*, and *F*) shows that the APC induced reduction in β-catenin protein (Fig. 2, *A* and *B*) is reversed by proteasomal inhibitors ALLN (Fig. 2, *C* and *D*) and lactacystin-β lactone (Fig. 2,*E* and *F*).

APC Down-regulates WT β-Catenin but Not the Non-ubiquitinatable S37A Mutant Form of β-Catenin-induced LEF Signaling—Mutation of a single serine residue (S37A) within the ubiquitination-targeting sequence prevents β-catenin ubiquitination (25). Serine mutations in the ubiquitin-targeting sequence of β-catenin occur in a number of different cancers (33–38). At least one of these, S37A, is a stabilizing mutation that renders β-catenin resistant to ubiquitination (25). If indeed APC regulates β-catenin-LEF signaling by targeting β-catenin for proteasomal degradation, then it should not be able to down-regulate the non-ubiquitinatable S37A mutant β-catenin protein or the LEF signaling induced by this stable form of β-catenin. To test this hypothesis, vector, FLAG-tagged WT, or S37A mutant β-catenin constructs were cotransfected with vector or WT APC and the LEF reporters into SW480 cells. β-Catenin-LEF signaling was monitored by assaying LEF reporter activity. Overexpression of both WT and S37A mutant forms of β-catenin increased the basal LEF reporter activity by about 30%, even against the background of high levels of en-

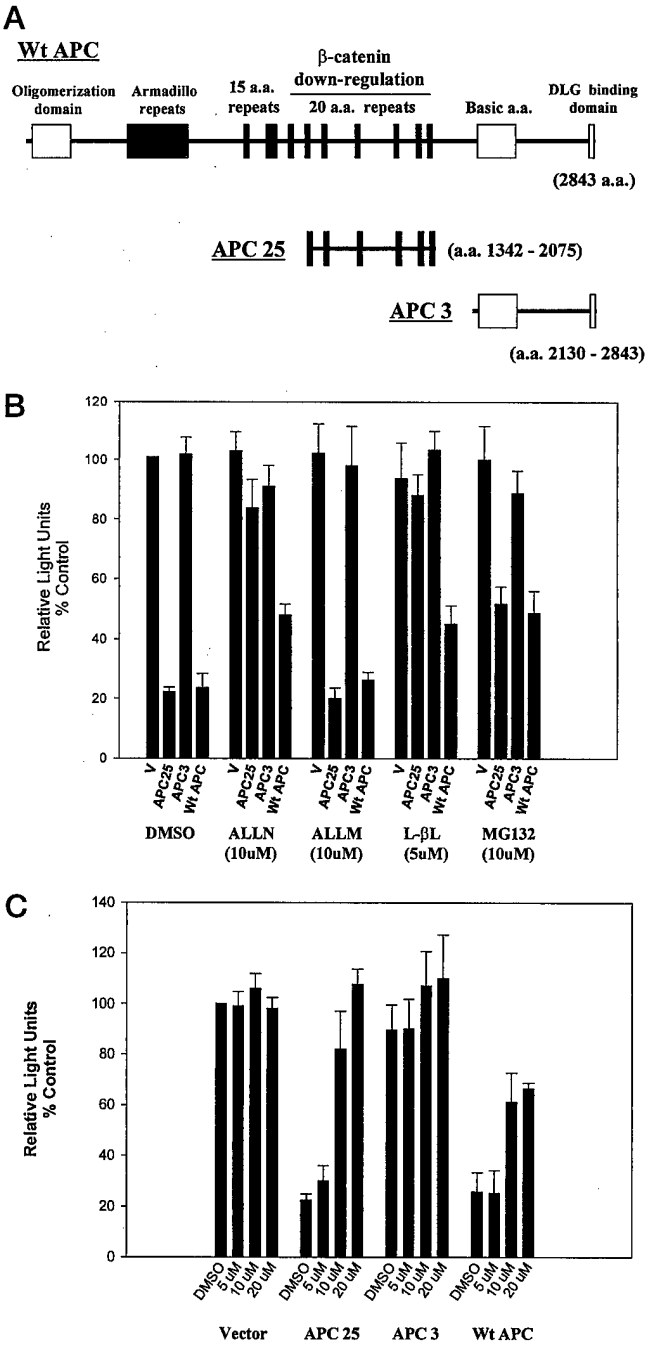

FIG. 1. *A*, the structure of WT APC and APC deletion constructs (26); *B*, APC-mediated down-regulation of β-catenin- LEF signaling is reversed by proteasomal inhibitors. SW480 cells were transiently transfected with various APC constructs, using LipofectAMINE-Plus reagent (Life Technologies, Inc.). 12 h posttransfection, the cells were treated with proteasomal inhibitors ALLN, lactacystin-β lactone, and MG-132 or with Me_2SO (*DMSO*, vehicle) and ALLM (calpain inhibitor II) for 12 h. β-Catenin-LEF signaling was assayed using the LEF reporters pTOPFLASH (and pFOPFLASH; data not shown) (31). Raw data were normalized for transfection efficiency and potential toxicity of treatments, using pCMV-*Renilla* luciferase and the dual luciferase assay system (Promega). The experiment was repeated at least three times, with each treatment repeated in triplicate. *Error bars* represent S.D. *C*, APC-mediated down-regulation of β-catenin-LEF signaling is reversed by the proteasomal inhibitor, ALLN, in a dose-dependent manner. The transfections were performed as described in *B* and were followed by treatment with the various doses (μM) of the proteasomal inhibitor, ALLN. *a.a.*, amino acid(s); *DLG*, Discs Large protein.

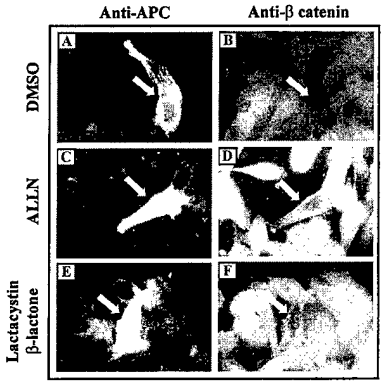

FIG. 2. **APC-mediated down-regulation of β-catenin protein is reversed by proteasomal inhibitors.** SW480 cells were transfected with WT APC and treated with Me_2SO (*DMSO*, *A* and *B*), 10 μM ALLN (*C* and *D*), or 5 μM lactacystin-β lactone (*E* and *F*). Double immunofluorescent staining for APC (*A*, *C*, and *E*) and β-catenin (*B*, *D*, and *F*) was performed according to Munemitsu *et al.* (11, 40).

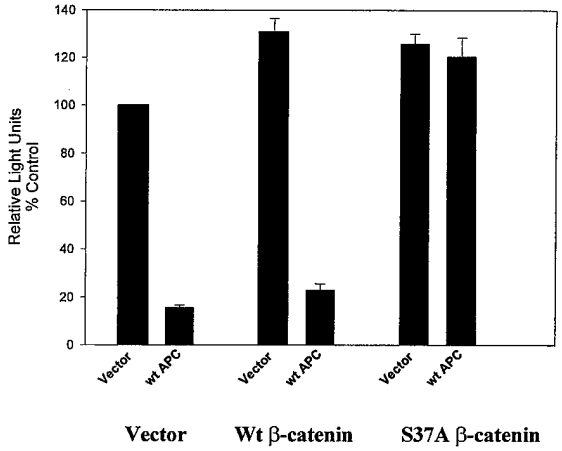

FIG. 3. **APC down-regulates LEF signaling induced by WT β-catenin but not by the non-ubiquitinatable S37A mutant β-catenin.** SW480 cells were transfected with empty vector or FLAG-tagged WT β-catenin or FLAG-tagged S37A β-catenin and empty vector or WT APC constructs, LEF reporters, and pCMV-*Renilla* luciferase. 24 h posttransfection, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

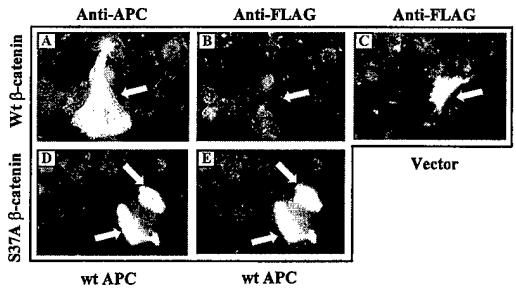

FIG. 4. **APC down-regulates WT β-catenin but not the non-ubiquitinatable S37A mutant β-catenin protein.** SW480 cells were transfected with FLAG-tagged WT β-catenin (*A*, *B*, and *C*) or FLAG-tagged S37A β-catenin (*D* and *E*) and WT APC constructs (*A*, *B*, *D*, and *E*) or empty vector (*C*). Double immunofluorescent staining for APC (*A* and *D*) and β-catenin (*B*, *C*, and *E*) were performed according to Munemitsu *et al.* (11, 40), except that the tranfected FLAG-tagged β-catenin was detected using anti-FLAG antibodies (Kodak).

dogenous β-catenin and β-catenin-LEF signaling in the SW480 cells. S37A β-catenin is more stable than WT β-catenin (in cells that actively degrade β-catenin, *e.g.* SKBR3 cells), but both forms increased LEF signaling by comparable levels in SW480 cells (which lack the ability to degrade β-catenin). Fig. 3 shows that APC down-regulates LEF signaling induced by WT β-catenin but not by the S37A mutant β-catenin. The ability of APC to down-regulate the cotransfected FLAG-tagged WT β-catenin and the S37A β-catenin protein levels was examined by double immunofluorescent staining using anti-APC antibodies and anti-FLAG antibodies (Kodak) (40). By double immunofluorescent staining for both the FLAG epitope and APC, we were able to monitor effects of APC specifically on the coexpressed forms of β-catenin. Fig. 4*A* (anti-APC) and Fig. 4*B* (anti-FLAG) show that WT APC effectively down-regulates WT β-catenin. Fig. 4*C* (anti-FLAG) shows that in concurrent transfections with empty vector and FLAG-tagged WT β-catenin, the FLAG-tagged WT β-catenin is expressed and the anti-FLAG antibody efficiently detects it. Fig. 4, *D* and *E* shows that APC does not down-regulate the S37A mutant β-catenin protein. These findings complement the observations of Munemitsu *et al.* (41) and Li *et al.* (42) that APC associates with but does not down-regulate β-catenin with an N-terminal deletion.

The Bisindoylmaleimide-type PKC Inhibitor GF-109203X Decreases the Ability of APC to Down-regulate LEF Signaling in a Dose-dependent Manner—PKC activity is required for Wnt-1 growth factor signaling to inhibit GSK-3β activity (18). TPA-induced down-regulation of diacylglycerol (DAG)-dependent PKCs prevents Wnt from inhibiting GSK-3β (18). However, our earlier studies demonstrate that neither the PKC inhibitor calphostin C nor TPA-induced down-regulation of PKCs stabilizes β-catenin (25). In contrast, the bisindoylmaleimide-type PKC inhibitor GF-109203X causes a dramatic accumulation of β-catenin in the cytoplasm (25). The bisindoylmaleimides inhibit both DAG-dependent and -independent PKC isoforms by competing with ATP for binding to the kinase, whereas calphostin C and long term TPA treatment inhibit only DAG-dependent PKC activities. The inhibitor profile implicates DAG-independent, atypical PKC activity in regulating β-catenin stability. These kinase(s) may offer a level of regulation distinct from the DAG-dependent PKC isoforms that regulate Wnt-dependent and GSK-3β-mediated β-catenin signaling (25).

The bisindoylmaleimide-type PKC inhibitor GF-109203X prevents β-catenin ubiquitination but does not inhibit GSK-3β (25). We tested the hypothesis that GF-109203X will inhibit the ability of APC to regulate β-catenin-LEF signaling. Fig. 5 shows that the PKC inhibitor GF-109203X decreases the ability of APC to down-regulate LEF signaling in a dose-dependent manner in SW480 cells. The changes in β-catenin-LEF signaling are paralleled by changes in β-catenin protein (Fig. 6). Similar results were obtained with another bisindoylmaleimide-type PKC inhibitor Ro31-8220 (data not shown).

Lithium (Li^+) Does Not Inhibit the Ability of APC to Down-regulate β-Catenin-LEF Signaling—Physiologically effective concentrations of Li^+ specifically and reversibly inhibit GSK-3β activity *in vitro* and *in vivo* and can mimic the effects of Wnt signaling on β-catenin in mammalian cells (43–46). Treatment of breast cancer cell lines with lithium results in the accumulation of the cytoplasmic signaling pool of β-catenin (25). Axin, the recently described product of the mouse *Fused* locus, forms a complex with GSK-3β, β-catenin, and APC (47). Axin promotes GSK-3β-dependent phosphorylation of β-catenin and may therefore help target β-catenin for degradation (48). However, overexpression of Axin inhibits β-catenin-LEF signaling in SW480 colon cancer cells in the absence of functional, WT APC. It is not known if APC promotes GSK-3β-dependent phosphorylation of β-catenin. Rubinfeld *et al.* (49) have shown that the APC protein is phosphorylated by GSK-3β *in vitro* and suggest that this phosphorylation event is linked to β-catenin turnover. It has also been suggested that APC and Axin may regulate the degradation of β-catenin by different mechanisms (50).

We tested the hypothesis that Li^+ can inhibit the ability of

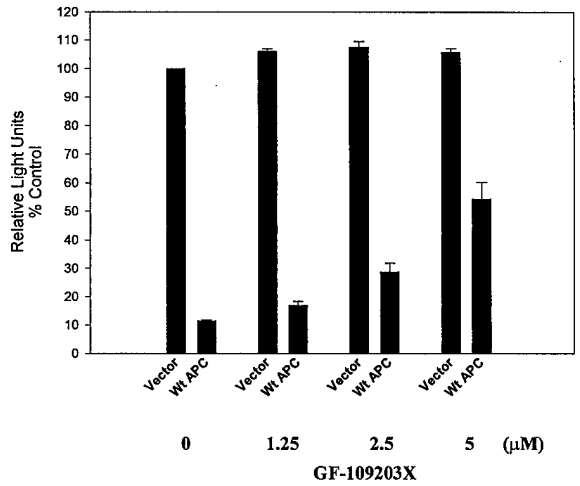

FIG. 5. **The bisindoylmaleimide-type PKC inhibitor, GF-109203X, which prevents β-catenin ubiquitination, inhibits APC-mediated down-regulation of β-catenin-LEF signaling in a dose-dependent manner.** SW480 cells were transfected with empty vector or WT APC, LEF reporters, and pCMV-*Renilla* luciferase. 12 h posttransfection, cells were treated with various concentrations of GF-109203X. 12 h later, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

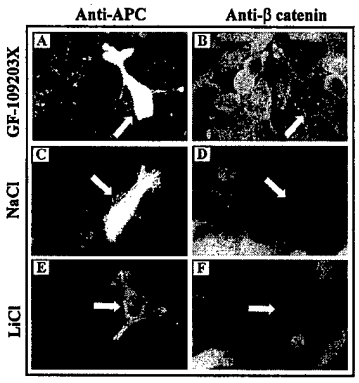

FIG. 6. **The bisindoylmaleimide-type PKC inhibitor, GF-109203X, but not lithium, reverses the APC-mediated down-regulation of β-catenin protein.** SW480 cells were transfected with WT APC and were treated with 5 μM GF-109203X (*A* and *B*) for 12 h as described in Fig. 5. 20 mM NaCl (*C* and *D*) or LiCl (*E* and IF) were added immediately following transfections and were present throughout the 24-h assay period to assure GSK-3β repression. Double immunofluorescent staining for APC (*A*, *C*, and *E*) and β-catenin (*B*, *D*, and *F*) was performed according to Munemitsu *et al.* (11, 40).

APC to down-regulate β-catenin-LEF signaling. The colon cancer cell line SW480 was transfected with empty vector or WT APC and treated with 10, 20, or 40 mM LiCl or NaCl for 24 h. The treatments were initiated immediately following the 5-h transfection period, and the cells were exposed to LiCl or NaCl throughout the 24-h assay period to assure GSK-3β repression. Fig. 6 shows that lithium does not alter the ability of WT APC to down-regulate β-catenin protein. Fig. 7 shows that lithium does not reverse the ability of WT APC to down-regulate LEF reporter activity in SW480 cells. Even at 40 mM lithium, a level well above that required to completely inhibit GSK-3β, exogenous WT APC continues to significantly down-regulate LEF reporter activity. These experiments were repeated in several different formats incorporating variations in the amount of WT APC transfected, duration of treatment with lithium, and timing of treatment initiation following transfections. Regardless of these variations, lithium does not inhibit the ability of exogenous APC to down-regulate β-catenin-LEF signaling in the colon cancer cells tested. Lithium treatment also leads to activation of AP-1-luciferase reporter activity in *Xenopus* embryos,

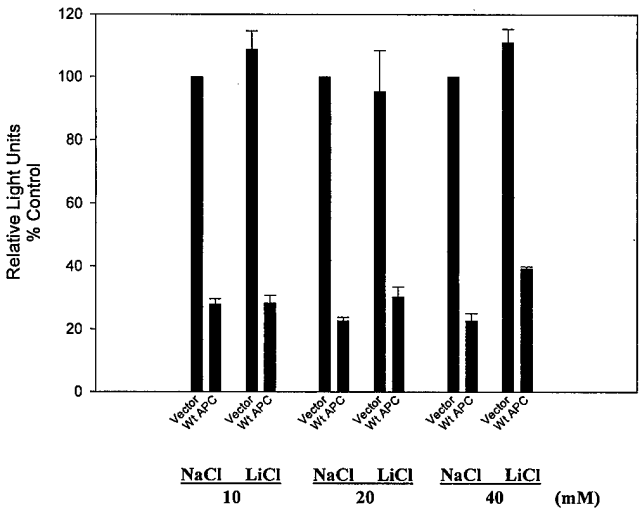

FIG. 7. **Lithium, an inhibitor of GSK-3β, does not significantly alter the ability of exogenous WT APC to down-regulate LEF reporter activity.** SW480 cells were transfected with empty vector or WT APC, LEF reporters, and pCMV-*Renilla* luciferase. Various concentrations of NaCl or LiCl were added immediately after transfection to assure GSK-3β repression. 24 h later, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

consistent with previous observations that GSK-3β inhibits c-*jun* activity (46, 51). Concurrent AP-1 transactivation assays also confirmed that GSK-3β was inhibited in SW480 cells following treatment with lithium (data not shown). These results indicate that GSK-3β activity (the molecular target of lithium action, in the Wnt signaling cascade) is not required for the ability of exogenously expressed APC to down-regulate β-catenin. Recent data indicated that the role of GSK-3β may be to potentiate assembly of the APC·Axin·β-catenin complex (48). In our experiments, the high level of APC expressed in the transiently transfected cells may well drive complex assembly in the absence of GSK-3β activity. Indeed, in SKBR3 cells, lithium treatment causes the accumulation of cytoplasmic β-catenin and increases β-catenin-LEF signaling[2] (25).

Our observations suggest that one function of APC is to down-regulate β-catenin-LEF signaling via the ubiquitin-proteasome pathway. *In vitro* reconstitution experiments designed to explore β-catenin ubiquitination suggested the requirement of key components other than GSK-3β and APC.[2] During the course of this study there has been an explosion of data describing novel proteins, including Axin, Conductin, and Slimb·β-TrCP as regulators of β-catenin stability (47, 52–57). In *Drosophila,* loss of function of Slimb results in accumulation of high levels of Armadillo and the ectopic expression of Wg-responsive genes (56). Recently, the receptor component of the IκB·ubiquitin ligase complex has been identified as a member of the Slimb·β-TrCP family (39). Considering the increasing number of similarities between the regulation of IκB and β-catenin (25), it is tempting to speculate that like IκB, β-catenin ubiquitination occurs in a multiprotein complex that includes kinases, ubiquitin-conjugating enzymes, and co-factors. Context-dependent potentiation of this complex by GSK-3β and other serine kinase(s) may be regulated by DAG-dependent and -independent PKC activity, respectively. The challenge for future studies will be to determine the exact role of APC in this process.

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[2] V. Easwaran and S. Byers, unpublished observations.

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